INVESTIGATORS' REPORT

1,3-Butadiene: Cancer, Mutations, and Adducts

Part V: Hemoglobin Adducts as Biomarkers of 1,3-Butadiene Exposure and Metabolism

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ABSTRACT

1,3-Butadiene (BD)* is an important chemical used largely in the manufacture of synthetic rubber and thermoplastic resins. In addition, it has been identified in cigarette smoke, automobile exhaust, and gasoline vapor. The objective of this research was to develop highly sensitive and specific assays for the detection and quantitation of hemoglobin adducts of three BD metabolites: 1,2-epoxy-3-butene (BDO), 1,2,3,4-diepoxybutane (BDO_2), and 1,2-dihydroxy-3,4-epoxybutane (BDO-diol).

We have successfully developed an assay for both *N*-(2-hydroxy-3-butenyl)valine (HBVal) and *N*-(2,3,4-trihydroxybutyl)valine (THBVal) in hemoglobin. The six adducts measured were the two diastereomers (isomers I and II) of HBVal and the four diastereomers of THBVal (isomers I through IV, which were eluted as three peaks, 1, 2, and 3). HBVal and THBVal were measured in control and exposed $B6C3F_1$ mice and Sprague-Dawley rats (1,000 ppm BD for 13 weeks at 6 hours/day, 5 days/week). In a second set of animal exposures, total THBVal was determined in $B6C3F_1$ female mice ($n = 5$) exposed to 1,250 ppm BD for 1, 5, or 10 days (6 hours/day, 5 days/week). THBVal adducts were also monitored in occupationally exposed Chinese workers and nonoccupationally exposed U.S. laboratory workers.

This study utilized the modified Edman degradation method of Törnqvist and colleagues (1986). Briefly, the samples were subjected to Edman degradation, Centricon-30 ultrafiltration, washing on C_{18} columns, and acetylation for isomers of THBVal only, followed by gas chromatography–mass spectrometry (GC-MS) quantitation.

For the HBVal assay, an authentic internal standard globin alkylated with [$^2H_6$]BDO was used; for the THBVal assay, a synthesized external standard, THB[$^{13}C_5$]Val, was used after Edman degradation.

The mean ± SD amounts of total HBVal measured in exposed mice (in pmol/g globin) were 16,560 ± 3,910 for female mice ($n = 4$) and 12,400 ± 2,030 for male mice ($n = 5$). The corresponding values for rats were 8,690 ± 930 for female rats ($n = 5$) and 5,480 ± 2,880 for male rats ($n = 3$). The total amount of THBVal (eluted peaks 1, 2, and 3) in male mice ($n = 5$) was 78,900 ± 13,700; and in females ($n = 2$) was 56,100 ± 100. In male rats ($n = 3$), the detected value was 9,650 ± 1,620 and in females ($n = 3$) the value was 21,600 ± 6,780. In control male mice ($n = 4$), the total level of THBVal isomers was ~27 pmol/g globin. In a control male rat, total THBVal was ~15 pmol/g globin.

In the time course study, the amount of THBVal adducts increased linearly with exposure, resulting in values of 4,200 ± 830, 19,760 ± 1,780, and 35,940 ± 3,460 pmol/g globin following 1, 5, or 10 days of exposure to 1,250 ppm BD, respectively.

Detection of HBVal in human samples was difficult due to low concentrations of adducts and a high background in the chromatograms. In a pooled sample from 4 individuals, we performed multiple separations with high-pressure liquid chromatography (HPLC) of the derivatized adducts and detected 4.6 pmol/g globin (that is, 2.7 and 1.9 pmol/g globin for isomers I and II, respectively). We measured the amounts of THBVal in both

* A list of abbreviations appears at the end of the Investigators' Report.

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nonoccupationally exposed U.S. laboratory workers and occupationally exposed workers from a polybutadiene plant in China. The mean total amount of THBVal among the U.S. laboratory workers was 36 ± 23 pmol/g globin for nonsmokers (n = 7) and 40 ± 9 for smokers (n = 4), compared with a mean total amount of 39 ± 13 pmol/g globin in a control set of Chinese workers (n = 25). These control values are overestimations of the true values because the amounts of THBVal in globin samples from other unexposed individuals (15 of 51) were below our limit of detection. BD-exposed Chinese workers had a total amount of 88 ± 59 pmol/g globin THBVal. The difference between smokers and nonsmokers was not significant, whereas the difference between control and exposed Chinese workers was highly significant ($p < 0.001$).

These data demonstrate that the methods developed in this project have adequate sensitivity to permit human biomonitoring for BD-hemoglobin adducts.

INTRODUCTION

1,3-Butadiene is an important industrial chemical used largely in the manufacture of synthetic rubber (namely, styrene-butadiene rubber and polybutadiene rubber) and thermoplastic resins (namely, acrylonitrile-butadiene-styrene). The annual production volume of BD in the United States is approximately 3 billion pounds, and worldwide annual production is approximately 12 billion pounds (Morrow 1990). BD has been identified in cigarette smoke, automobile exhaust, and gasoline vapor. Low levels of BD (0.5 to 10 ppb) have been detected in ambient air in some urban locations. Industrial production and use are estimated to account for 1.6% of environmental BD with mobile emissions representing 78.8% and miscellaneous sources accounting for 19.6% (U.S. Environmental Protection Agency 1994). Approximately 52,000 American workers are considered to be possibly exposed to BD (National Institute for Occupational Safety and Health 1990). A comprehensive review of BD toxicology and epidemiology was published by Himmelstein and colleagues (1997).

CARCINOGENICITY

1,3-Butadiene is carcinogenic in mice and rats following chronic inhalation exposure (National Toxicology Program 1984, 1993; Owen et al. 1987). Significant species differences exist in carcinogenic potency. Mice develop increased neoplasia following exposures to 6.25 ppm BD and higher. Rats are much less susceptible to BD-induced carcinogenesis, but they have been evaluated only at exposures of 1,000 or 8,000 ppm BD. The major differences in carcinogenic potency between rats and mice are associated with clear differences in metabolism (Himmelstein et al. 1997).

Until recently, epidemiologic studies have provided few consistent data on the carcinogenic risks of BD for humans (International Agency for Research on Cancer 1987). A comprehensive study on 15,649 BD workers by Delzell and colleagues (1996) has shown that exposures in the styrene-butadiene rubber industry cause higher incidences of leukemia with increasing exposure and duration of employment. In contrast, increases in leukemia incidence have not been shown in workers exposed to BD monomer. Instead, a few studies have found increased incidences of lymphosarcoma, but only in workers with short-term exposure. In these same cohorts, workers with exposure at the same time, but continuing for long term, did not show increases in the incidence of lymphosarcoma. Other individuals, including monomer workers in the Delzell study, did not have an excess in incidence of either lymphosarcoma or leukemia (Divine and Hartman 1996). Presently, the data support a conclusion that the styrene-butadiene rubber manufacturing process is carcinogenic for humans, but the evidence for BD monomer is limited.

METABOLISM

A simplified metabolic scheme for BD is shown in Figure 1. Three electrophilic metabolites are formed: BDO, BDO_2, and BDO-diol. BDO represents the initial metabolite formed by cytochrome P450 2E1 (*CYP2E1*) oxidation. BDO can undergo detoxification by glutathione or epoxide hydrolase. The latter reaction produces 1,2-dihydroxy-3-butene (BD-diol), which can be conjugated by glutathione to form the urinary metabolite 1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane (MI) (Sabourin et al. 1992; Bechtold et al. 1994). BD-diol can undergo a second oxidation to form BDO-diol. Likewise, BDO can be further metabolized by *CYP2E1* to form BDO_2, which in turn can be hydrolyzed by epoxide hydrolase to BDO-diol. Several investigators have shown that mice produce much greater amounts of BDO_2 than do rats. Metabolism studies on BD using human liver suggest that humans are more like rats than mice in the type and amount of metabolites formed; however, considerable interindividual variation in humans was noted (Seaton et al. 1995; Boogaard and Bond 1996; Boogaard et al. 1996).

GENOTOXICITY

The genotoxic effects of BD are considered to be due to the reaction of its three major metabolites, BDO, BDO_2, and

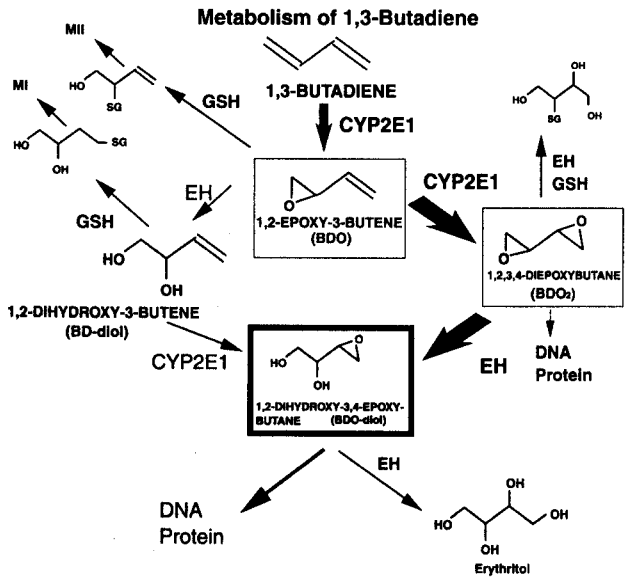

Figure 1. BD Metabolism. The three structures enclosed in boxes represent electrophiles that can covalently bind to the *N*-terminal valine of hemoglobin. MI = 1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane, and MII = 1-hydroxy-2-(*N*-acetylcysteinyl)-3-butene (both urinary metabolites of BD); GSH = reduced glutathione; and EH = epoxide hydroxylase.

BDO-diol, with the nucleophilic centers in DNA. BDO_2 is nearly 100 times more mutagenic in vitro in human TK6 lymphoblasts than is either BDO or BDO-diol (Cochrane and Skopek 1994). BDO_2 is also much more toxic than the other epoxides. Furthermore, nearly half of the mutations induced by BDO_2 are large deletions, whereas such deletions only occur at background frequencies in BDO-exposed cultures. BD is also genotoxic in vivo, inducing micronuclei in mice (Adler et al. 1994; Autio et al. 1994; Stephanou et al. 1997; Xiao et al. 1996), but not in rats (Autio et al. 1994). It also induces heritable translocations in mice (Adler et al. 1995, 1998) and *hprt* mutations in rats and mice (Tates et al. 1994, 1998; Meng et al. 1996). BD has produced variable results in humans (Tates et al. 1996; Legator et al. 1993; Ward et al. 1994) and genetic effects in germ cells in mice (Brinkworth et al. 1998).

GENETIC POLYMORPHISMS

Several investigators have demonstrated that the null genotype for the polymorphisms in the glutathione *S*-transferase *T1* (*GSTT1*) and *M1* (*GSTM1*) loci confers increased genotoxic susceptibility to human lymphocytes exposed to BDO_2 and BDO, respectively (Wiencke et al. 1995; Uusküla et al. 1995; Norppa et al. 1995). It is also reasonable to expect that polymorphisms in the epoxide hydrolase and *CYP2E1* loci could affect the rates of detoxification and activation of BD and its metabolites.

DNA ADDUCTS

The identification and quantitation of DNA adducts formed by BD and its metabolites have been actively studied by several research teams during the past five years. DNA adducts have been characterized at the N7 position of guanine and the N1, N3, and N6 positions of adenine (Citti et al. 1984; Leuratti et al. 1994; Neagu et al. 1995; Tretyakova et al. 1996, 1997a,b,c, 1998; Koivisto et al. 1996, 1998). Multiple DNA adducts are formed at each of the above positions because attack at the C1 and C2 positions of BDO and at the C1 position of both BDO-diol and BDO_2 are possible. This is further complicated by diastereomers if nucleosides or nucleotides are being measured as well as by stereoisomers of the prime 2′,3′,4′-(trihydroxybut-1′-yl)-adenine and -guanine adducts formed by BDO-diol and racemic and *meso*-BDO_2.

HEMOGLOBIN ADDUCTS

Measurements of hemoglobin adducts offer some important advantages over other measurements of internal dose, including the following: (a) Blood samples are readily available for epidemiologic and biomonitoring studies and contain large amounts of hemoglobin. (b) The analysis of protein adducts is highly sensitive and specific. Hemoglobin adducts are not repaired, and they thus accumulate for the lifespan of the erythrocyte, allowing studies to be carried out at low exposure levels. (c) Studies of hemoglobin adducts in humans can provide considerably improved exposure assessments because they integrate exposure over time. (d) Adducts provide a measure of the dose of reactive chemicals in laboratory animals and in humans, thereby eliminating some of the difficulties in extrapolating from high to low doses and across species (Osterman-Golkar et al. 1993).

The BD metabolite BDO has been shown to react with hemoglobin to form HBVal adducts (Osterman-Golkar et al. 1991, 1993). Two major and two minor peaks were identified with use of GC-MS. The two major peaks were shown to be the diastereomers resulting from attack on the *N*-terminal valine-NH_2 at the C1 position of BDO. Adduct concentrations of 1 to 3 pmol/g globin were recorded in nonsmoking persons working in a production area with ~1 ppm BD levels. Adducts also were measured in cigarette smokers who were not occupationally exposed to BD. The reported adduct levels were lower in humans than in mice and rats exposed to 2 ppm BD and were also lower than hydroxyethylvaline adducts associated with

occupational exposures to ethylene oxide and ethylene. Albrecht and colleagues (1993) reported adduct amounts five times higher in mice than in rats (17 and 3.5 nmol/g globin, respectively, after exposure to 500 ppm, 6 hours/day, for 5 days), although the diastereomers were not resolved. It is clear that BD exposure results in a supralinear dose response that is characteristic of saturation of metabolic activation and that mice have higher amounts of monoepoxide adducts than do rats (Albrecht et al. 1993; La and Swenberg 1997). This conclusion is also supported by two studies on the induction of micronuclei in rats and mice (Adler et al. 1994; Autio et al. 1994). In pilot studies, we compared male and female rats and mice exposed to 1,000 ppm BD for 13 weeks and found that females had higher levels of BDO-hemoglobin adducts than did males (Tretyakova et al. 1996). All of the hemoglobin studies have utilized the modified Edman degradation method of Törnqvist and colleagues (1986), which is based on GC-MS measurements using an internal standard of [$^2H_4$]-*N*-(2-hydroxyethyl)valine ([$^2H_4$]HEVal), [^{14}C]-*N*-(2-hydroxypropyl)valine, or *N*-(2-hydroxy-butenyl)valine-glycine-glycine.

At the time this project was initiated, there were no reports of measurements of the hemoglobin adducts of BDO-diol or BDO_2. In view of the greater formation of BDO_2 by the mouse compared with formation in the rat, and the much greater mutagenicity of BDO_2, it was important that methods be developed so that both quantitative and relative comparisons of hemoglobin adducts could be made between species. During the conduct of this project, Pérez and colleagues (1997) reported formation of THBVal adducts in hemoglobin of rats and humans. The THBVal adducts were formed in greater amounts than the previously measured levels of HBVal adducts. The authors concluded that BDO-diol appeared to be an important metabolite of BD.

SPECIFIC AIMS

The objective of this research was to develop high- resolution mass spectrometric methods for quantitating the hemoglobin adducts of BD and to utilize these methods to analyze globin samples from mice, rats, and humans exposed to BD. The long-term goals of this research were to better understand the mechanisms of BD carcinogenicity and to improve the accuracy of cancer risk assessment for BD. The adducts studied include those formed by BDO, BDO_2, and BDO-diol with the *N*-terminal valine of globin.

METHODS AND STUDY DESIGN

This research project was designed to develop new or improved methods for measuring the number and type of hemoglobin adducts in blood from laboratory animals and humans using GC-MS techniques.

SPECIMENS AVAILABLE FOR ANALYSIS

Washed red blood cell (RBC) samples were available for species comparisons between rats and mice. These samples had been stored at –70°C for 5 years and had been obtained from male and female Crl:CD®BR rats and B6C3F_1/CrlBR mice exposed by inhalation to 1,000 ppm BD (6 hours/day, 5 days/week, for 13 weeks) at the Haskell Laboratory for Toxicology and Industrial Medicine, E. I. duPont de Nemours and Company (Newark, DE). The animals had been fed Certified Purina Laboratory Chow #5002 and had water available ad libitum. These samples were expected to have steady-state levels of HBVal and THBVal adducts because the exposure period exceeded the life span of the erythrocytes. An additional set of mouse blood samples (which had been stored for 1 year) was available from B6C3F_1 female mice exposed to 1,250 ppm for 1, 5, or 10 days (6 hours/day, 5 days/week) at the Chemical Industry Institute of Toxicology (Research Triangle Park, NC). These animals were fed Lab-Blox chow and had water ad libitum except during exposures. Based on published data regarding BD metabolism and hemoglobin and DNA adducts, the 1,250 ppm concentration of BD saturates the metabolism of BD to its epoxide intermediates. Therefore, the 13-week exposure samples should have the highest number of BD adducts possible.

For human studies, we had 87 coded samples of RBCs from workers in a polybutadiene plant in China. The set included samples from both exposed and unexposed individuals and constituted a small portion of the analyses conducted in a large molecular epidemiologic study (Hayes et al. 1996). As such, the samples provided molecular dosimetry data on hemoglobin adducts to compare with exposure data, *hprt*$^-$ mutations, and other measured biomarkers. Institutional Review Board approval was obtained at the time the specimens were collected; we were exempt from the need for further approval because we had no information that identified the sampled individuals. After analysis of THBVal adducts, the data were sent to Dr. Hayes for decoding and comparing with exposure measurements. In addition, the number of THBVal adducts in humans were compared with those found in BD-exposed rats and mice. The study had limited ability to demonstrate whether or not large interindividual differences in adduction exist because only limited exposure

data were available. Finally, the study provided information on the limits of detection of hemoglobin adducts that will be useful for designing future molecular epidemiologic studies of occupational and environmental exposure. During the evaluation of samples from Chinese workers, we obtained 13 additional blinded human samples from laboratory volunteers at the University of North Carolina to compare with the samples obtained in China.

CHEMICALS

BD (99% pure) and racemic mixtures of BDO (98% pure) and BDO_2 (97% pure) were purchased from Aldrich (Milwaukee, WI). [$^2H_6$]BD (98% enriched) was obtained from Cambridge Isotopes (Andover, MA). Pentafluorophenylisothiocyanate (PFPITC) and formamide were purchased from Fluka (Buchs, Switzerland). The [$^2H_4$]HEVal globin standard was provided by John MacNeela from the Chemical Industry Institute of Toxicology. HBVal, HB[$^{13}C_5$]Val, THBVal, and THB[$^{13}C_5$]Val were synthesized by Dr. J. Krzeminski from American Health Foundation under contract from the National Cancer Institute. All other reagents and solvents used were analytic-reagent or HPLC grade.

BLOOD SAMPLE STORAGE

Samples of saline-washed RBCs from rats, mice, and humans were frozen in liquid nitrogen and stored at −70°C until analyzed. This period of time ranged from 1 to 5 years for rodent samples and was 3 years for the Chinese human samples.

ISOLATION OF GLOBIN

Frozen washed RBCs from mice, rats, and humans were thawed and diluted in equal volumes of distilled deionized water (DDW). Globin was isolated according to the method of Mowrer and colleagues (1986), as modified slightly by Walker and colleagues (1993) for mouse globin. DDW (1 mL) was added to 1 mL of washed packed RBCs, and the tubes were inverted gently to ensure complete mixing. After the mixing was complete, the solution was allowed to sit for several minutes, and then 50 mM HCl in 2-propanol was added (6 mL for rat globin, 9 mL for mouse globin, and 8 mL for human globin). The tubes were mixed thoroughly, employing a vortex at a setting of 2 or 3, and then centrifuged at 4°C at 1,500 × *g* for 30 minutes. The supernatant was carefully decanted into a fresh tube, and 4 mL ethyl acetate was added for rat samples, 6 mL for mouse samples, and 5 mL for human samples. The pellet was discarded. The ethyl acetate-supernatant mixture was vortexed thoroughly until the solution turned cloudy with globin precipitate. The samples were centrifuged at 4°C at 1,500 × *g* for 5 minutes, the supernatant discarded, and the globin pellet saved. Ethyl acetate (4 mL) was added to resuspend the pellet. The tube was centrifuged at 4°C at 1,500 × *g* for 5 minutes and the supernatant discarded. This step was repeated until the supernatant was colorless. Then the pellet was resuspended in 4 mL of *n*-pentane, centrifuged, and the supernatant discarded. The final pellet was dried carefully under a gentle stream of nitrogen gas. After the pellet was dry, the pellet was transferred to a clean, labeled screw-cap vial. The vials (loosely capped) were placed in a vacuum desiccator under house vacuum for several hours or overnight, and then they were placed under a stronger vacuum for several more hours. The samples were then derivatized or stored in vials (tightly capped) at − 70°C.

DERIVATIZATION OF GLOBIN SAMPLES

All glassware used for sample derivatization and cleanup was silanized in a 5% solution of dimethyldichlorosilane in *n*-pentane, rinsed twice with methanol, and air dried. The derivatization was performed according to the *N*-alkyl Edman procedure, based on Törnqvist's modified Edman degradation for specific cleavage of *N*-alkylated terminal valines of the four chains in hemoglobin (Törnqvist 1986). Routinely, 2 to 5 mg mouse globin, 6 to 10 mg rat globin, or 50 to 250 mg human globin was dissolved in 1.5 mL formamide (volume for up to 50 mg globin). A few further modifications to the method included the addition of 20 µL 1M NaOH/mL formamide to each sample and the addition of 10 µL PFPITC/50 mg globin to the human samples. All samples were shaken overnight (about 18 hours) at room temperature (25°C) and then for 1.5 hours at 45°C. The samples were filtered through Centricon-30 columns, the retentate was discarded and the filtrate used.

HBVal Adducts

An internal standard of rat globin treated with [$^2H_6$]BDO (see Standards section) and containing 48 pmol *N*-terminal [$^2H_6$]HBVal/mg globin (total for both diastereomers) was added to each sample. After completion of the Edman degradation, the samples were extracted three times with 2 mL diethyl ether, dried under nitrogen gas, and then redissolved in toluene. The samples were washed: first with 2 mL DDW, next with 2 mL freshly prepared 0.1 M sodium carbonate (Na_2CO_3), and then with 2 mL DDW. The toluene layer was evaporated under nitrogen gas, and the samples were then reconstituted in 50 µL dry toluene for GC-MS analysis.

Method I for THBVal Adducts

For rodent samples, 5 to 15 mg globin was used, whereas 100 to 150 mg globin was used for all human samples. After completion of the Edman degradation, an external standard of THB[$^{13}C_5$]Val-pentafluorothiohydantoin (PFPTH) was added, and samples were filtered through Centricon-30 columns. The rodent samples were extracted three times with 3 mL ether, dried under nitrogen gas, and then redissolved in toluene and washed with 1 mL 0.1 M Na_2CO_3 followed by 1 mL DDW to minimize loss due to analyte solubility in aqueous solutions. For the human samples, after evaporating the pooled ether extractions, the residue was dissolved in 200 µL formamide and applied to prepared 500-mg C_{18} columns (50:50 formamide:DDW), and washed sequentially with 1 mL each of DDW, 0.1 M Na_2CO_3, and DDW. After drying the columns, the samples were eluted in 4 mL dry acetonitrile and evaporated under nitrogen gas. The dry samples were acetylated with 25% triethylamine and 25% acetic anhydride in acetonitrile for 30 minutes at room temperature (25°C), dried, resuspended in *n*-pentane, and washed with 3 mL 60% aqueous methanol. The *n*-pentane layer was evaporated and resuspended in 20 to 50 µL toluene for GC-MS analysis. The samples were stable and could be stored for several months at –20°C.

Method II for THBVal Adducts

For rodent samples, 5 to 15 mg globin was used; for all human samples, 100 to 150 mg globin was used. After completion of the Edman degradation, an external THB[$^{13}C_5$]Val-PFPTH standard was added, and samples were filtered through Centricon-30 columns. The samples were extracted three times with 3 mL diethyl ether and dried under nitrogen gas. The residue was dissolved in 1 mL fresh 0.1 M Na_2CO_3 and applied to prepared 100-mg C_{18} columns (50:50 formamide:DDW), and washed with 1 mL DDW. After drying the columns, the samples were eluted in 3 mL dry acetonitrile and evaporated under nitrogen gas. The dry samples were acetylated with 25% triethylamine and 25% acetic anhydride in acetonitrile for 30 minutes at room temperature (25°C), dried, resuspended in 3 mL *n*-pentane, and washed with 2 mL 60% aqueous methanol. The *n*-pentane layer was evaporated and resuspended in 20 to 50 µL toluene for GC-MS analysis. The samples were stable and could be stored for several months at –20°C.

LIQUID CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS

The technique of liquid chromatography–electrospray ionization–mass spectrometry (LC-ESI-MS) (negative mode) was conducted on synthesized standards using a Pharmacia liquid chromatographic system with dual pumps (#2248, Pharmacia LKB Biotechnology, Uppsala, Sweden) coupled to a Finnigan 4000 quadrupole mass spectrometer (Finnigan-MAT, San Jose, CA) that was retrofitted with a pneumatic electrospray source (Analytica of Branford, Branford, CT). The LC column used was C_{18} (150 mm × 0.8 mm internal diameter; Hypersil, 3 µm particle size, LC Packings, San Francisco, CA). The flow rate was 22 µL/minute. The LC separation was performed by the following program: 50% to 100% aqueous methanol for 10 minutes, 100% from 10 to 20 minutes, and 100% to 50% from 20 to 35 minutes.

GAS CHROMATOGRAPHY–MASS SPECTROMETRY ANALYSIS

Samples were analyzed by GC-MS in the electron-capture negative chemical ionization (ECNCI) mode with methane as reagent gas (3×10^{-5} mbar) and using a VG 70-250SEQ hybrid mass spectrometer with a Hewlett-Packard 5890 GC. The GC separation was performed on an Alltech, SE-54 column (30 mm × 0.32 mm, 1.0 µm film thickness; Alltech Associates, Deerfield, IL) with 10 psi He head pressure and 300°C injector temperature. Injections were made in the direct mode with the following column temperature program: 100°C to 300°C at the rate of 10°C per minute. The instrument was operated at 70 eV and emission current at 500 µA. The source temperature was ~170°C and 250°C during HBVal and THBVal analysis, respectively. Full-scan mass spectra were obtained at a resolving power of 1,000. Quantitative selected ion monitoring (SIM) measurements were performed at a resolving power of 10,000. The ions monitored were at a mass-to-charge ratio (*m/z*) of 318.0450 and 320.0573 (for HBVal) and 534.1084 and 539.1254 (for THBVal) for the analyte and the external standard, respectively. Three replicates were analyzed from each rodent sample. Duplicate samples were analyzed from humans if enough globin was available. Perfluorokerosene was used for tuning and calibrating the mass spectrometer (Ranasinghe et al. 1998).

STANDARDS

To generate an internal standard of [$^2H_6$]HBVal globin, the following procedure was used: A solution containing 20 mg [$^2H_6$]BDO in 7 mL acetone synthesized and quantitated according to Sangaiah and colleagues (1997) was added to 2 mL freshly washed and lysed RBCs. The mixture was incubated at 37°C for 3 hours and the globin was isolated according the method of Mowrer and colleagues (1986).

The four standards of HBVal-PFPTH, HB[$^{13}C_5$]Val-PFPTH, THBVal-PFPTH, and THB[$^{13}C_5$]Val-PFPTH were prepared from adducted valine standards synthesized by Dr. Krzeminski from the American Health Foundation. Synthesis was conducted according to the procedure of Törnqvist (1994) and had more than 90% yield. The HBVal-PFPTH and HB[$^{13}C_5$]Val-PFPTH standards were prepared by dissolving HBVal or HB[$^{13}C_5$]Val in 3 mL 0.5 M potassium bicarbonate ($KHCO_3$) and 1.5 mL 1-propanol; 10 µL PFPITC (pH 8) was added, and the solution was incubated for 3 hours at 45°C. This was extracted two times with 2 mL heptane and evaporated under nitrogen gas. The THBVal-PFPTH and THB[$^{13}C_5$]Val-PFPTH standards were prepared by dissolving THBVal or THB[$^{13}C_5$]Val in 1 mL 0.5 M $KHCO_3$ and 0.5 mL 1-propanol; 10 µL PFPITC (pH 8) was added, and the solution was incubated for 2 hours at 45°C, after which 1.0 mL DDW was added. This was extracted four times with 3 mL ether, evaporated under nitrogen gas, dissolved in 3 mL toluene, washed in 1 mL 0.1 M Na_2CO_3 and 1 mL DDW, and dried again under nitrogen gas.

The isomers of the analytes and internal standards were dissolved in formamide and separated by HPLC using UV detection (268 nm). For primary separation, a semipreparative column (Beckman Ultrasphere C_{18}, 25 cm × 0.60 cm, 5 µm) was used with a linear gradient of 10% to 100% methanol in water over 20 minutes. The fractions of interest from multiple injections were pooled together and further purified on an analytical column (Ultrasphere C_{18}, 25 cm × 0.46 cm, 5 µm) using the same mobile-phase and gradient program. All separated isomers were quantitated by UV absorption at 268 nm. The standards were further characterized by GC-MS in ECNCI mode and by LC-ESI-MS full-scan spectra.

CALIBRATION

The calibration solutions were prepared by dissolving the standards in 1.5 mL formamide containing control rat globin (10 mg per sample) as a protein carrier. In the case of HBVal, we added 0.45 mg internal standard globin with [$^2H_6$]HBVal and different amounts of globin with HBVal, which were obtained by in vitro alkylation of RBCs with unlabeled BDO as described previously. Then the solutions were processed using the same protocol as for sample preparation. Two separate calibration curves were built for both diastereomers. The synthesized HBVal-PFPTH and HB[$^{13}C_5$]Val-PFPTH (latter added after the Edman degradation step) were also used as reference standards for stability control of the internal standard [$^2H_6$]HBVal globin.

In the case of THBVal, 1.5 mL formamide samples containing control rat globin (10 mg per sample) as a protein carrier were subjected to Edman degradation. Different amounts of the THBVal-PFPTH standard and 8 pmol of THB[$^{13}C_5$]Val-PFPTH external standard were added to the samples, and the calibration solutions were processed by the same protocol as for sample preparation. Separate calibration curves were built for the three diastereomer peaks of THBVal as measured by GC-MS.

QUALITY ASSURANCE

The laboratory worked with the intent of Good Laboratory Practices. Each of our staff was required to keep a laboratory notebook acceptable to Good Laboratory Practices standards. These notebooks were archived in a central location when full and not in use. Samples were stored in a freezer at –70°C until processed. The GC-MS instrumentation was carefully tuned and calibrated before use; each calibration was noted and a known solution similar to the samples was run to check for day-to-day reproducibility. A synthesized derivatized standard was run every fifth sample to ensure run-to-run reproducibility and stability of calibration.

STATISTICAL ANALYSES

The coefficient of variation for assay performance was determined using one-way analysis of variance (ANOVA). Comparisons of adduct concentrations between (1) male and female rats, (2) male and female mice, (3) male mice and male rats, (4) female mice and female rats, and (5) all mice and all rats were made by ANOVA followed by the Tukey-Kramer multiple-comparisons test. A value of $p < 0.05$ was required for statistical significance. Comparisons of THBVal between BD-exposed and unexposed controls in the Chinese human molecular epidemiologic study were made by the t test, as was the comparison between THBVal values of smokers and nonsmokers. A value of $p < 0.05$ was required for statistical significance. Least-squares regression analysis was used to compare THBVal and measurements of environmental exposure. Values are reported as means ± SD.

RESULTS

MASS SPECTROMETRY

Standards

The synthesized standards HBVal-PFPTH and THBVal-PFPTH, as well as the corresponding [$^{13}C_5$]-labeled external standards, were separated by HPLC as described above

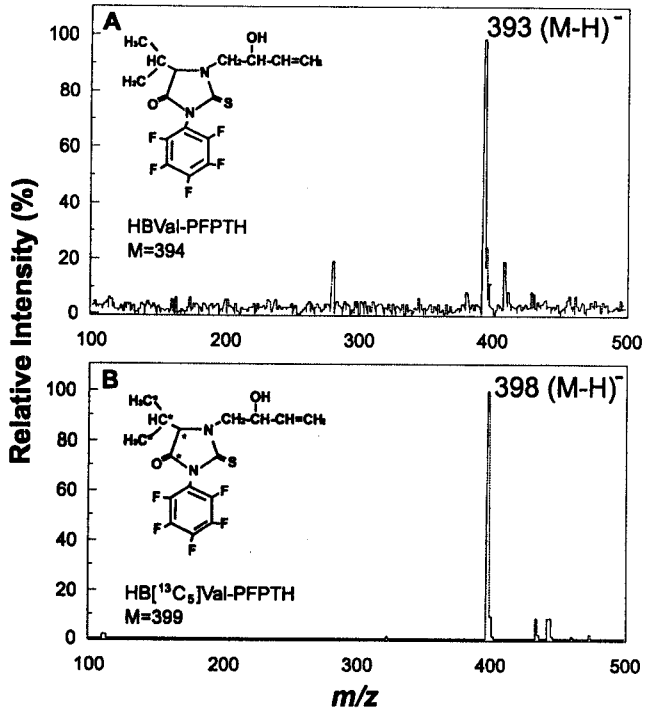

Figure 2. LC-ESI-MS (negative mode) spectra of (A) HBVal-PFPTH and (B) HB[$^{13}C_5$]Val-PFPTH.

in the Standards section. Figure 2 illustrates the LC-ESI-MS (negative mode) spectra for isomer I of HBVal-PFPTH (Figure 2A) and HB[$^{13}C_5$]Val-PFPTH (Figure 2B), demonstrating the most abundant ions $(M–H)^-$ at *m/z* 393 and 398 for the analyte and external standard, respectively. A similar full scan could not be run on the [$^2H_6$]HBVal globin. Figure 3 represents full-scan GC-ECNCI-MS spectra of isomer I of HBVal-PFPTH (Figure 3A), [$^2H_6$]HBVal globin (Figure 3B), and HB[$^{13}C_5$]Val-PFPTH (Figure 3C). There were no differences in the fragmentation patterns in the full-scan MS spectra for the diastereomers. The main fragment ions in the GC-ECNCI-MS spectra for HBVal-PFPTH were *m/z* 374 [M–HF] and *m/z* 318 [M–CH_2=CH–CHO]. The corresponding ions for the deuterated internal globin standard were *m/z* 380 and *m/z* 320, whereas those for the [$^{13}C_5$] external standard were at *m/z* 379 and *m/z* 323. Under our conditions, the highest sensitivity was achieved by SIM of the ions at *m/z* 323, 320, and 318. We attempted tandem mass spectrometry (MS/MS) of *m/z* 374 to 318 (*m/z* 380 to 320, respectively, for the deuterated internal globin standard), but the sensitivity was about 20 times less compared with direct SIM of [M–HF], although the background level was substantially decreased.

The LC-ESI-MS (negative mode) spectra for THBVal-PFPTH (Figure 4A) and the [$^{13}C_5$]-labeled external standard

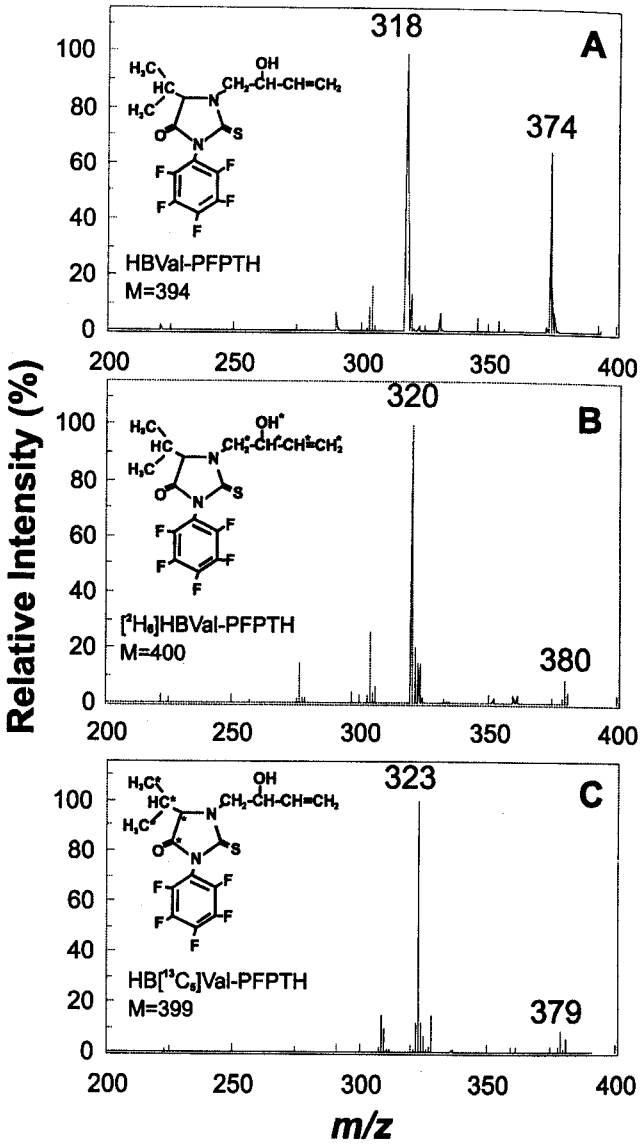

Figure 3. Full-scan GC-ECNCI-MS spectra of (A) HBVal-PFPTH, (B) [$^2H_6$]-HBVal globin, and (C) HB[$^{13}C_5$]Val-PFPTH.

(Figure 4B) demonstrate $(M–H)^-$ peaks at *m/z* 427 and 432, re- spectively. The full-scan GC-ECNCI-MS spectra of THBVal-PFPTH (after acetylation) and THB[$^{13}C_5$]Val-PFPTH (peak 3, after acetylation) are shown in Figure 5. For the GC-MS analysis of THBVal-PFPTH and THB-[$^{13}C_5$]Val-PFPTH, the dominant fragment ions were *m/z* 534 and 539, respectively, corresponding to [M–HF]. The isotopic labeling pattern of the ions helps to elucidate the fragmentation mechanism of the molecular ions during ECNCI processes. The common ion, *m/z* 451, is due to the loss of the valine group and HF (M–HF–C_5H_7O). The ions *m/z* 534 and *m/z* 323 (Figure 5A) originate from the loss of HF and the acetylated butadiene group [$C_4H_6(OAc)_3$],

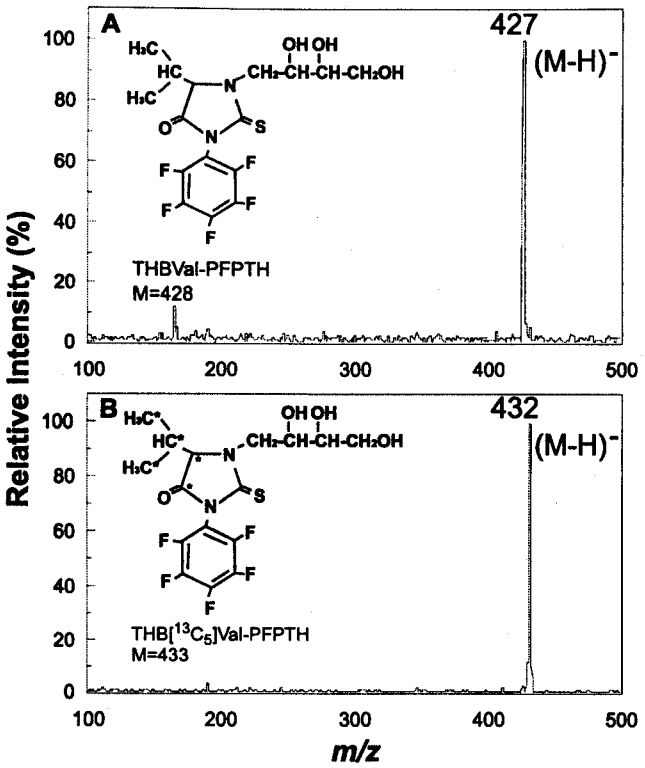

Figure 4. LC-ESI-MS (negative mode) spectra of (A) THBVal-PFPTH and (B) THB[$^{13}C_5$]Val-PFPTH.

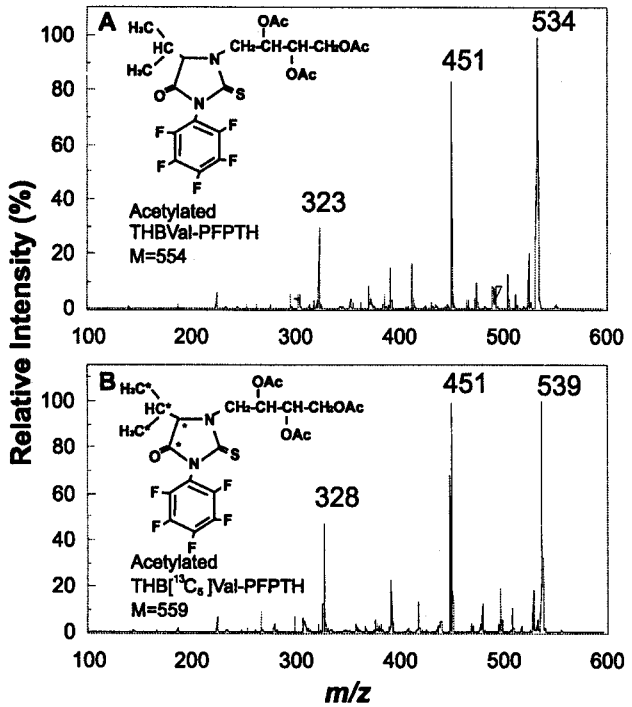

Figure 5. Full-scan GC-ECNCI-MS spectra of (A) THBVal-PFPTH and (B) THB[$^{13}C_5$]Val-PFPTH.

respectively. This is confirmed by the presence of all of the ^{13}C labels, as indicated by the 5 amu mass shifts in the corresponding fragment ions, *m/z* 539 and 328 (Figure 5B). There were relatively low backgrounds in the THBVal chromatograms at these SIMs.

Calibration Curves

The calibration curves for both isomers (namely, I and II) of HBVal globin versus the internal standard [$^2H_6$]- HBVal globin are shown in Figure 6. The corresponding calibration curves for THBVal-PFPTH versus THB[$^{13}C_5$]Val-PFPTH (diastereomer peaks 1, 2, and 3) are presented in Figure 7. All calibration curves gave a linear response in the concentration range used for quantitation.

PERFORMANCE OF VARIOUS METHODS

During the course of methods development and sample analysis, it became apparent that both the HBVal globin standard and the corresponding [$^2H_6$]HBVal globin internal standard degraded when stored at –20°C, but not at –70°C. To establish the amount of [$^2H_6$]HBVal in the alkylated globin, it was compared with an [$^2H_4$]HEVal globin standard containing 16.3 pmol/mg globin. Three independent experiments, analyzing 5 to 6 replicates each, were performed, and the values of 27.65 ± 2.04 pmol/mg globin for isomer I and 20.36 ± 1.83 pmol/mg globin for isomer II of [$^2H_6$]HBVal were obtained. To control for standard degradation, synthesized HBVal-PFPTH and its [$^{13}C_5$]-labeled standard were also used to check globin standard concentrations. The reported HBVal measurements were based on a deuterated internal globin standard that had been calibrated using standard curves constructed with [$^2H_4$]HEVal.

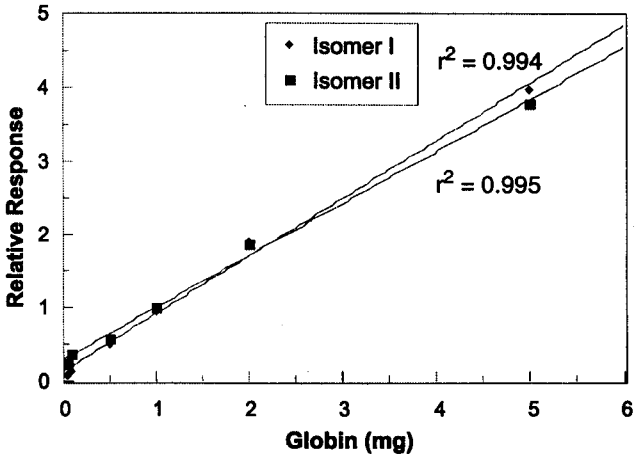

Figure 6. Calibration curves for HBVal-PFPTH (isomers I and II) versus [$^2H_6$]HBVal-PFPTH.

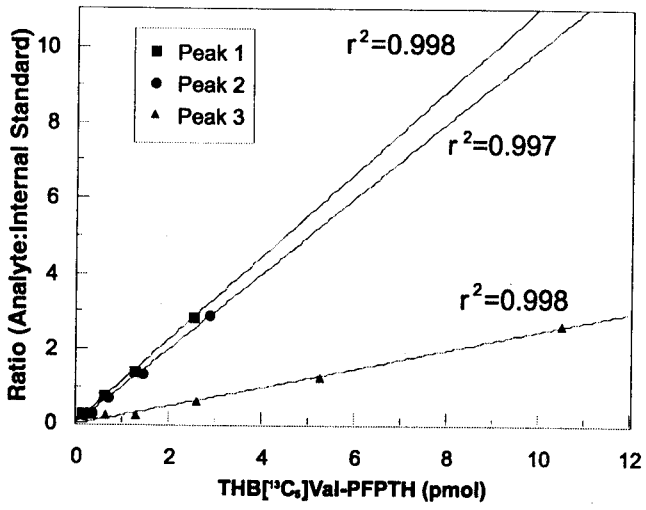

Figure 7. Calibration curves for THBVal-PFPTH (peaks 1, 2, and 3) versus THB[$^{13}C_5$]Val-PFPTH.

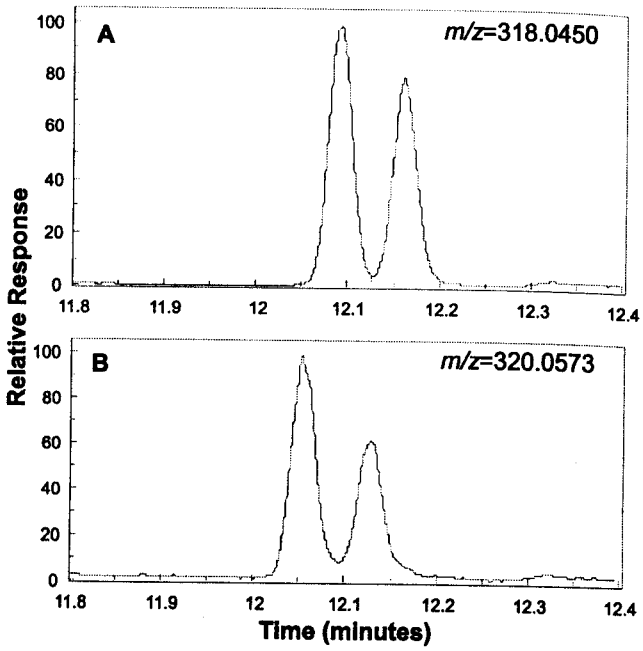

Figure 8. GC-ECNCI-MS chromatograms of (A) HBVal in a globin sample from a male rat exposed to 1,000 ppm BD for 13 weeks (9 mg globin) and of (B) the internal standard, [$^2H_6$]HBVal globin. In each chromatogram, isomer I is the first peak and isomer II is the second, smaller peak.

For quantitation of THBVal in the rodent and human samples noted in this report, the [$^{13}C_5$]-labeled external standard for THBVal was added after the Edman degradation because this reaction is considered to be very reproducible and of high yield (Törnqvist 1986; Bergmark 1997). THBVal adducts in globin appeared to be stable when stored at –70°C. Samples of rat and human globin were analyzed by different individuals more than a year apart with good agreement in analysis results.

The recovery of analytes after sample preparation was monitored by adding, at the end of the procedure, synthesized standards HBVal-PFPTH or THBVal-PFPTH to calibration solutions that contained known amounts of both analyte and internal standard. The total recovery was established to be 10% to 12% in both assays. Most of the loss occurred during the clean-up steps.

Reproducibility of the method for total HBVal was evaluated using triplicate globin samples (including two isomers) from 17 animals from the 13-week, 1,000 ppm BD exposure. The coefficient of variation was ± 9%; however, interindividual variability was greater. Reproducibility of the assay for total THBVal was established by repeated processing of 8 rodent globin samples from the 13-week, 1,000 ppm BD exposure. These latter samples were processed up to 16 months apart by two different individuals. The coefficient of variation for these assays was ± 14%.

ANALYSIS OF HBVal AND THBVal IN RATS AND MICE EXPOSED TO BD

A representative HBVal GC-ECNCI-MS chromatogram of a globin sample from a rat exposed to 1,000 ppm BD for 13 weeks is shown in Figure 8. The chromatogram shows two HBVal peaks (isomers I and II, resulting from attack at the C1 position) with slightly different retention times for the analyte and the deuterated internal globin standard. The ratio between the intensity of the two peaks varied from 1.2 to 1.6. The natural isotopic contribution of nondeuterated HBVal to the deuterated internal standard (2%) was taken into account and the corresponding corrections made when necessary.

The data for HBVal adducts in mice and rats exposed to 1,000 ppm BD for 13 weeks (6 hours/day, 5 days/week) revealed about twice as many adducts in mice than in rats ($p < 0.001$; Table 1). Although females of both rodent species appeared to have higher numbers of HBVal adducts than males, the difference did not reach statistical significance.

The GC-ECNCI-MS chromatogram of THBVal from an exposed rat (1,000 ppm BD, 13 weeks at 6 hours/day, 5 days/week) is presented in Figure 9, together with a chromatogram of the THB[$^{13}C_5$]Val-PFPTH external standard. The three peaks correspond to the diastereomeric THBVal generated by BDO-diol and BDO_2. Peaks 1 and 2

Table 1. HBVal from Red Blood Cells from Mice and Rats Exposed to 1,000 ppm BD[a]

Animals (*n*)	Isomer I	Isomer II	Total
Mice			
Males (5)	7,020 ± 1,240	5,430 ± 790	12,400 ± 2,030
Females (4)	9,600 ± 2,070	6,970 ± 1,930	16,560 ± 3,910
Rats			
Males (3)	3,150 ± 1,560	2,340 ± 1,320	5,480 ± 2,880
Females (5)	4,990 ± 560	3,700 ± 420	8,690 ± 930

[a] Exposures were 6 hours/day, 5 days/week, for 13 weeks. Values are in pmol HBVal/g globin, and expressed as means ± SD.

correspond to the first peak published by Pérez and colleagues (1997), whereas peak 3 corresponds to the second peak monitored by the same authors.

The data for THBVal measured in exposed mice and rats are presented in Table 2. The concentration of THBVal adducts in female mice was ~2.5 times greater than that in female rats ($p < 0.05$), whereas male mice had ~8 times more THBVal than did male rats ($p < 0.001$). Likewise, THBVal was greater in all mice than in all rats ($p < 0.001$). Two comparisons, of male rats with female rats and male mice with female mice, showed no significant differences.

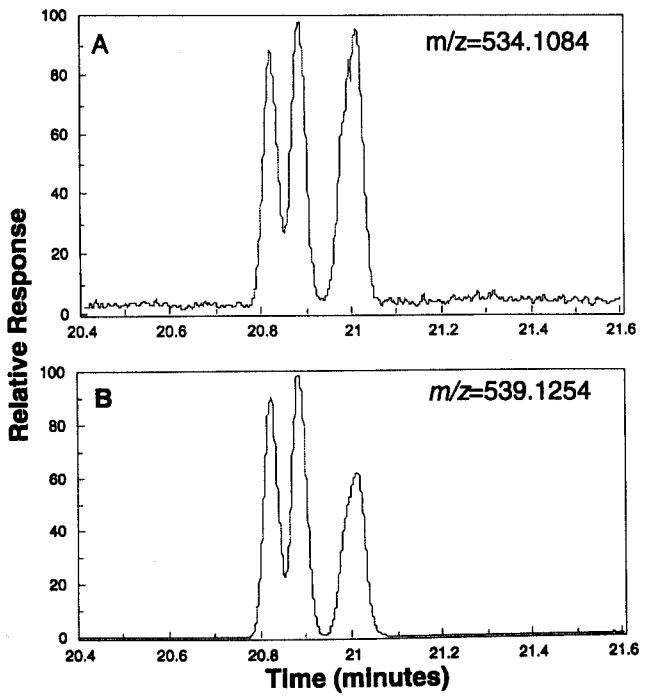

Figure 9. GC-ECNCI-MS chromatograms of (A) THBVal in a globin sample from a male rat exposed to 1,000 ppm BD for 13 weeks (6 mg globin) and of (B) the external standard, THB[$^{13}C_5$]Val-PFPTH. In each chromatogram, the first peak is isomer I, the second peak is isomer II, and the third peak contains isomers III and IV.

Table 2. THBVal from Red Blood Cells from Mice and Rats Exposed to 1,000 ppm BD[a]

Animals (*n*)	THBVal
Mice	
Males (5)	78,900 ± 13,700
Females (2)	56,100 ± 100
Rats	
Males (3)	9,650 ± 1,620
Females (3)	21,600 ± 6,780

[a] Exposures were 6 hours/day, 5 days/week, for 13 weeks. Values are in pmol THBVal/g globin, and expressed as means ± SD.

The THBVal adducts were also detectable in samples from a control male rat (15 pmol/g) and a pooled sample from four male mice (27 pmol/g), although much larger amounts of globin were needed for these analyses (80 to 120 mg). Thus, male mice and rats exposed to 1,000 ppm BD for 13 weeks had THBVal adducts that were ~3,000- and ~650-fold greater than levels in controls, respectively. When the data from Tables 1 and 2 are compared, the ratio of total THBVal to HBVal was 6.3 for male mice, 3.4 for female mice, 1.8 for male rats, and 2.5 for female rats. In all cases where THBVal and HBVal data were collected on the same animals, THBVal levels were greater.

Globin samples from a second study with female mice exposed to 1,250 ppm BD for 1, 5, or 10 days were analyzed for total THBVal (Figure 10). A linear increase in THBVal was associated with increased BD exposure. Comparing these data with the above data on endogenous THBVal in male mice suggests that a single 6-hour exposure to 1,250 ppm BD results in a increase of ~150-fold in THBVal adducts.

ANALYSIS OF HUMAN SAMPLES

The initial analyses of human samples concentrated on HBVal measurements in samples obtained from the molecular epidemiologic study of Hayes and colleagues (1996). Analysis of human globin samples for HBVal used 15 to 50 mg globin and followed the standard protocol for sample preparation. This work failed to detect peaks of the analyte in any sample.

Unfortunately, repeated attempts to measure HBVal depleted eight of the samples from Chinese workers. There were two main reasons for the difficulty in detecting HBVal: The concentration of adducts in the samples was too low, below our detection limit, and the background of the chromatograms was too high, with a large interfering peak present just in front of the expected analyte peaks. In order to decrease the background level,

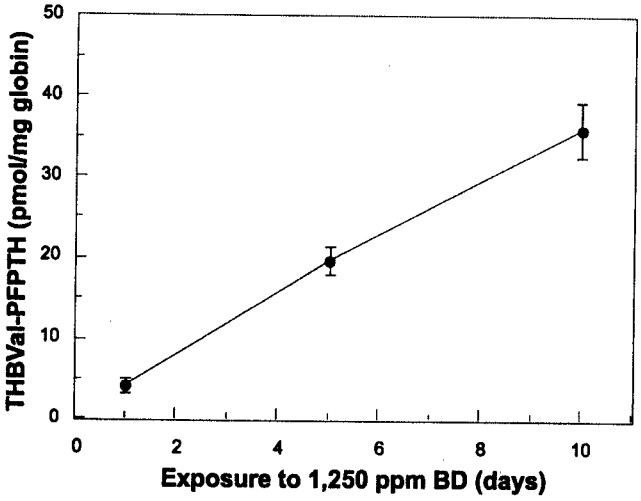

Figure 10. Relation between THBVal levels in female mice and duration of exposure to 1,250 ppm BD.

we tried to perform MS/MS (*m/z* 374 to 318). In one sample (54 mg globin) from a male worker in the recovery group, the peaks in the chromatogram were low, but after a smoothing operation we estimated the amount of HBVal to be 1.1 pmol/g globin for isomer I. The peak for isomer II was almost at the background level so no estimate could be made. This result demonstrated that MS/MS could lower the background. However, even for standards, the sensitivity of the MS/MS method was about 20 times lower than that of SIM high-resolution MS of *m/z* 318 and *m/z* 320 ions. The major reason for such poor sensitivity with MS/MS using this instrumentation appeared to be the extensive fragmentation that occurred in the source due to high temperature. This has not been a problem with other instruments (Ostermann-Golkar et al. 1991, 1993). A second attempt to measure HBVal used about 100 mg of pooled globin from four Chinese workers whose exposure groups were unknown to us. We performed multiple HPLC separations on the derivatized product using the same conditions as for standards (see Methods and Study Design). The process took several days, but we succeeded in monitoring HBVal at 2.8 and 1.9 pmol/g globin for isomers I and II, respectively, with large signal-to-noise ratios (Figure 11).

In contrast to HBVal, THBVal adducts were detectable in most human samples, including those from Chinese workers and from American volunteers (the latter including smokers and nonsmokers). The number of samples that had amounts of THBVal below our detection limit was greatest in the nonexposed (control) Chinese workers. Measurements could not be made in 6 of 24 female controls and 7 of 14 male controls. In contrast, only 8 of 41 workers had undetectable amounts of THBVal.

Figure 12 presents a representative chromatogram of a human globin sample of a nonexposed nonsmoker. Among samples from nonoccupationally exposed volunteers

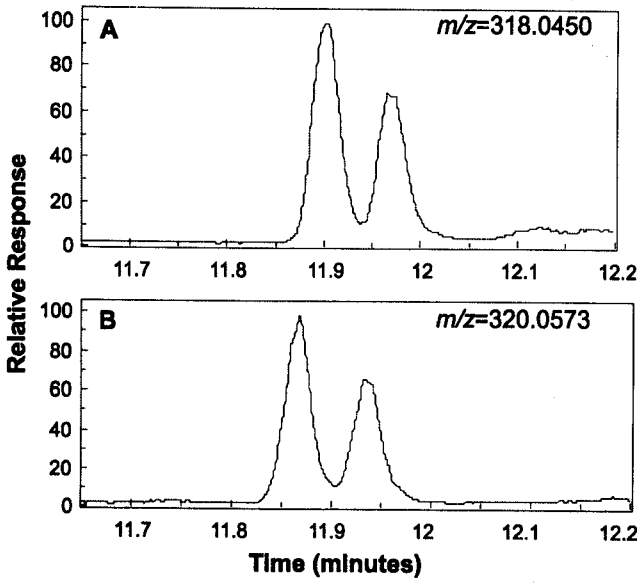

Figure 11. GC-ECNCI-MS chromatograms of (A) HBVal in a pooled blood sample from four Chinese workers and of (B) the internal standard, [$^2H_8$]-HBVal globin. Note that the peaks are similar to those shown in Figure 8.

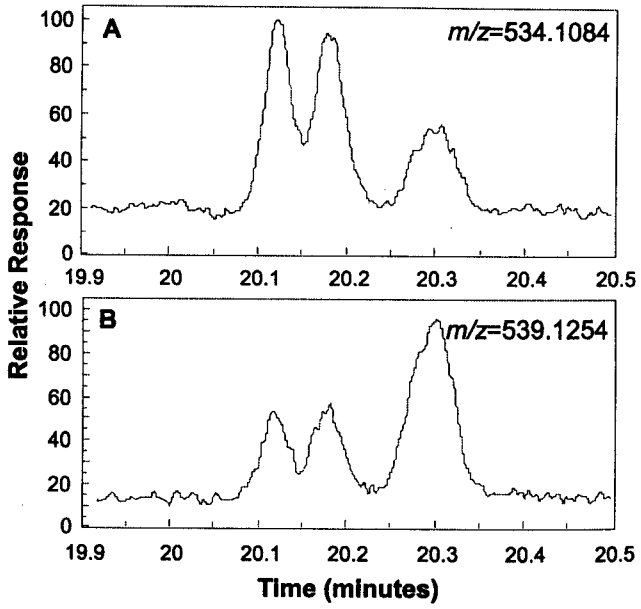

Figure 12. GC-ECNCI-MS chromatograms of (A) THBVal in a globin sample from an unexposed human (150 mg globin) and of (B) the external standard, THB[$^{13}C_5$]Val-PFPTH. In each chromatogram, the first peak is isomer I, the second peak is isomer II, and the third peak contains isomers III and IV.

(7 nonsmokers and 4 smokers), 11 of 13 samples had amounts of THBVal that were above our detection limit. The mean values for THBVal were 36 ± 23 pmol/g for nonsmokers and 40 ± 8 pmol/g for smokers. The difference between smokers and nonsmokers was not significant.

In the study of Chinese workers, we first analyzed the concentration of THBVal adducts in all individuals with or without potential occupational exposure to BD. The mean total amount of THBVal in 25 nonexposed workers was 39 ± 13 pmol/g globin, whereas the mean for 33 exposed workers was 88 ± 59 pmol/g globin. This difference was significant ($p < 0.001$). Male workers without occupational exposure to BD had values of 46 ± 20 pmol/g globin ($n = 7$), and female workers without occupational exposure had 37 ± 8 pmol/g ($n = 18$). The difference was not statistically significant. None of the females smoked, whereas 6 of the 7 males were smokers. The amount of THBVal in unexposed Chinese workers was not different from that of U.S. volunteers.

The THBVal data were then analyzed by job category (Hayes et al. 1996). Process analysts, whose job is to take BD samples from production process lines in either the polymerization or dimethyl formamide (DMF) facilities and then analyzed them by gas chromatography, had THBVal levels of 71 ± 24 ($n = 13$) and 140 ± 94 ($n = 7$) pmol/g globin, respectively. Figure 13 shows a chromatogram of THBVal adducts in globin from one of the DMF workers. Workers who did process control, minor maintenance, and when needed, major repairs had THBVal levels of 78 ± 48 ($n = 13$) pmol/g globin.

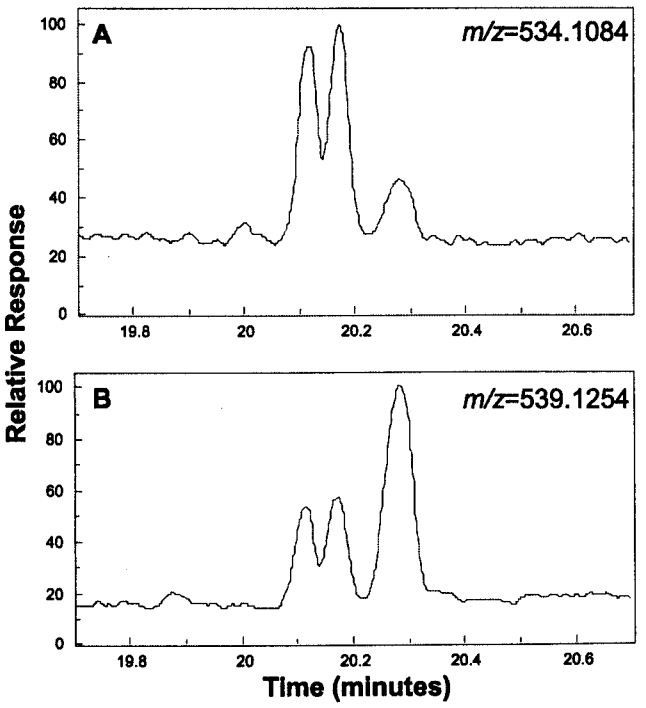

Figure 13. GC-ECNCI-MS chromatograms of (A) THBVal in a globin sample from a Chinese DMF worker (123 mg globin) and of (B) the external standard, THB[$^{13}C_5$]Val-PFPTH. In each chromatogram, the first peak is isomer I, the second peak is isomer II, and the third peak contains isomers III and IV.

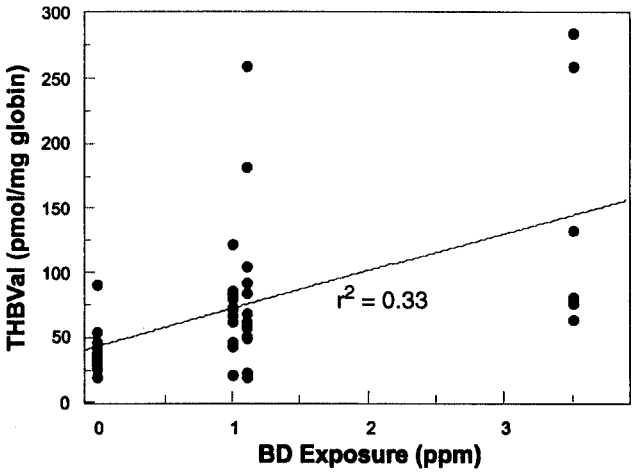

Figure 14. Relation between THBVal levels in Chinese workers and environmental air measurements of BD. The filled circles represent individuals. The nonexposed group (0 ppm BD) includes 25 workers, the polymerization group (1.0 ppm BD) includes 13 workers, the process control group (1.1 ppm BD) includes 13 individuals, and the DMF group (3.5 ppm BD) includes 7 workers.

Hayes and colleagues (1996) found that the median amount of BD in the environment (expressed as 6-hour time-weighted averages) of the polymerization workers was 1.0 ppm, whereas it was 3.5 ppm in the DMF work areas. The process analysts working on routine maintenance had median air concentrations of 1.1 ppm BD, but when pumps and related equipment were being repaired, the BD concentration was as high as 45 ppm. Figure 14 shows individual worker values for total THBVal plotted against these environmental values. The slope of the regression line was significantly different from zero ($p < 0.0001$) and demonstrated greater concentrations of THBVal adducts associated with increased BD exposure ($r^2 = 0.33$).

DISCUSSION

BD is an important industrial chemical used in the manufacture of synthetic rubber and thermoplastic resins and has a potential occupational exposure to over 50,000 American workers. In addition, BD is present in mobile-source emissions, cigarette smoke, and ambient air. In view of the clear carcinogenic potential of BD in laboratory animals, strong evidence of carcinogenic potential in humans during the manufacturing process of

styrene-butadiene rubber, and limited evidence of carcinogenic potential of BD monomer in humans, good measures of exposure must be obtained in order to assess accurately the human risk of cancer from BD exposure. Likewise, better indicators of which metabolites are formed and available to bind to DNA in humans and laboratory animals will promote a better understanding of critical mechanisms of action that need to be used for improved risk assessment.

With this understanding the present research project was undertaken. This laboratory has been one of the leading research groups investigating the DNA adducts formed by BD and the mutagenic consequences of such adduction. At the time this research was proposed, no methods had been developed to quantitate either DNA adducts or hemoglobin adducts of BDO_2. This represented a major gap in our understanding because BDO_2 had been shown to be much more mutagenic than either BDO or BDO-diol (Cochrane and Skopek 1994), yet all measures of BD-related adducts in DNA and hemoglobin had focused on BDO.

During the first year of the project, we applied published methods using a modified Edman degradation of the *N*-terminal valine (Osterman-Golkar et al. 1991, 1993) to the identification and quantitation of HBVal globin. This approach was successful in mice and rats exposed to high concentrations of BD (1,000 ppm, 13 weeks at 6 hours/day, 5 days/week). The long duration of exposure resulted in HBVal adducts that represented steady-state concentrations. $B6C3F_1$ mice had higher concentrations of HBVal adducts than did rats, but male mice (~12,400 pmol/g globin) did not differ significantly from female mice (~16,500 pmol/g globin). We found that male Sprague-Dawley rats had ~5,500 pmol/g globin and females had ~8,700 pmol/g. The higher numbers of adducts in females is consistent with the metabolism studies of Thornton-Manning and colleagues (1997). The lack of statistical significance between males and females may be due to the limited sample sizes. Previous studies of rats and mice have not used the same exposure regimen; however, in all studies mice have had higher numbers of HBVal adducts than rats. Osterman-Golkar and colleagues (1991) reported ~3,000 pmol HBVal adducts/g globin in male Wistar rats exposed to 1,000 ppm BD (2 weeks at 6 hours/day, 5 days/week). The same group reported ~900 pmol HBVal/g globin in male Sprague-Dawley rats exposed to 100 ppm for 4 weeks, and ~3,750 pmol/g in male $B6C3F_1$ mice. Albrecht and colleagues (1993) found ~300 pmol HBVal/g globin after exposure of female Wistar rats to 500 ppm BD for 5 days, and ~500 pmol/g after exposure to 1,300 ppm BD. The same authors reported ~1,700 and ~2,700 pmol/g globin in $CB6F_1$ mice exposed to the same concentrations of BD for 5 days (at 6 hours/day). Albrecht and colleagues (1993) were not able to resolve the two diastereomers of HBVal, whereas all of the other studies were based on combining the data for the two peaks.

In contrast to our success in measuring HBVal in highly exposed rodents, we were not able to detect this adduct in human samples. The problem stemmed from at least two factors. First, the concentration of HBVal in humans was much lower than that present in our BD-exposed rats and mice. Second, background was very high in the chromatograms, even when using high-resolution (10,000 ppm) MS. In the experiment using 100 mg of globin of a pooled sample from four Chinese workers whose exposure groups were unknown to us, we performed multiple HPLC separations on the derivatized product using the same conditions as for standards (see Methods and Study Design). The process took several days, but we succeeded in monitoring HBVal at 2.8 and 1.9 pmol/g globin for isomers I and II (see Figure 11). The resolution of these low concentrations was excellent. However, the method was completely unsuitable for routine analysis of human samples due to the time constraints associated with HPLC clean-up.

The high-resolution mass spectrometer that was used in our experiments was also capable of doing MS/MS analyses, so we attempted to measure one of the human samples by monitoring *m/z* 374 to 318 in order to reduce the background and to improve our signal-to-noise ratio. Although the background was dramatically reduced, we were unable to measure reliably the number of HBVal adducts. The major reason for this is thought to lie in the particular instrument used, which has a relatively hot source that caused fragmentation of the analyte as it came into the source. Thus, there was minimal *m/z* 374 available to undergo collision-induced dissociation to form *m/z* 318. Unfortunately, a number of the samples from Chinese workers were consumed during the HBVal studies and were not available for the THBVal studies. The limited HBVal data we did generate are consistent with HBVal data reported by others: that is, occupational exposures similar to those of the Chinese workers (1 to 3 pmol/g globin) (Osterman-Golkar et al. 1991).

During the last year of this project, we entered into a collaboration with Siv Osterman-Golkar to examine some samples from an independent study for THBVal. Dr. Osterman-Golkar had developed a GC-MS/MS method for THBVal that was later reported by Pérez and colleagues (1997). We made minor modifications to the method and applied high-resolution MS for quantitation. Whereas the GC-MS/MS method could only resolve the THBVal

stereoisomers into two peaks, we were readily able to resolve three peaks. The background in chromatograms was markedly lower than that experienced for HBVal. Furthermore, we found much higher amounts of THBVal in both animal and human samples than we had found for HBVal. In fact, it was possible to demonstrate THBVal in globin from unexposed rats, mice, and humans. Blood samples from a squirrel monkey, dog, calf, and horse were also examined and found to contain similar amounts of THBVal.

The number of THBVal adducts was highest in mice exposed to 1,000 ppm BD for 13 weeks, with females having ~56,000 pmol/g globin and males 79,000 pmol/g. Male rats had ~10,000 pmol/g globin, but female rats had ~22,000 pmol/g. Likewise, we were able to show clear increases in THBVal in female mice exposed to 1,250 ppm BD for 1, 5, or 10 days. There are presently no exposure-response data in rats or mice exposed to lower concentrations of BD. Molecular dosimetry studies on THB-guanine adducts in DNA of mice and rats exposed to 0, 20, 62.5, or 625 ppm BD for 4 weeks have demonstrated strong supralinear exposure-response curves (Koc et al. 1999). In contrast to the THB-guanine exposure-response data, BDO-guanine response was linear from 20 to 625 ppm BD. The number of THB-guanine adducts showed only small increases between 62.5 and 625 ppm BD in rats. In mice, the exposure response suggests biphasic formation, with a sharply increasing response between 0 and 62.5 ppm followed by a less steep slope, but not to the point of saturation of metabolic activation as in the rat. It is likely that a similar exposure response will follow for THBVal. Although the ratio of THBVal:HBVal adducts ranged between 2 and 3 in rats and 4 and 6 in mice exposed to 1,000 ppm BD, this may change markedly at lower exposures. The ratio of THB-guanine:BDO-guanine at 625 ppm was between 5 and 6 in rats and between 12 and 17 in mice, but at lower concentrations it ranged between 15 and 26 in rats and between 27 and 38 in mice.

This project clearly demonstrates the ability of THBVal to serve as a biomarker for human exposure to BD. Exposure-related increases in THBVal were demonstrated in the study of Chinese workers. The number of THBVal adducts was significantly different for all exposed versus unexposed individuals. In addition, the increases showed a good correlation with workplace exposure monitoring at levels ranging from 1 to 3.5 ppm BD.

Perhaps the greatest surprise was the demonstration of THBVal adducts in 70% of the unexposed individuals. The average number of adducts in U.S. volunteers was 36 pmol/g globin for nonsmokers, whereas for Chinese females with no exposure it was 37 pmol/g globin. Considering that 2 of the 13 volunteers and 6 of the 24 Chinese females were below the limit of detection, these averages are actually overestimates. Additional studies with adequate amounts of globin will be needed to depict fully the extent of interindividual variability. Nevertheless, it is highly likely that at least a part of these THBVal adducts arises from endogenous sources. Support in humans for this interpretation also comes from measurements of the MI metabolite in urine of unexposed individuals (Bechtold et al. 1994). This metabolite arises from glutathione conjugation with BD-diol (see Figure 1). Whether the source of BD-diol is from normal physiology or another endogenous chemical that forms THBVal and MI remains unknown. Support from animal studies for an endogenous source of THBVal adducts is provided by the finding of these adducts in six species other than humans. Ambient air concentrations of BD cannot be the source of the THBVal adducts in all of these cases because neither Chinese nor U.S. smokers had significantly different numbers of THBVal adducts. Furthermore, cigarette smoke contains much higher concentrations of BD than does ambient air, which is measured consistently at less than 1 ppb.

Pérez and colleagues (1997) suggest that THBVal arises predominantly from BDO-diol. They arrived at that conclusion in part by the observation that BDO_2 administration gave different THBVal adducts than did BDO-diol. A schematic in their paper, as well as the discussion about this point, were used to support this conclusion. Their schematic was incorrect in one regard, in that it did not show the *meso*-BDO_2. The BDO_2 that they administered consisted only of racemic BDO_2. *meso*-BDO_2 is clearly formed by human microsomes (Krause and Elfarra 1997) and would be expected to form the two stereoisomers that Pérez and colleagues (1997) only associated with BDO-diol exposure. Thus, there is no way to determine which contribution of THBVal comes from BDO-diol and which portion comes from BDO_2. Rydberg and colleagues (1996) have demonstrated the rapid formation of a cyclic adduct with BDO_2 and valinamide in vitro that would be specific for BDO_2. Unfortunately, this adduct cannot be analyzed using a modified Edman reaction and no other ultrasensitive method is presently known. The development of such a method would clearly be desirable because identification of the molecular dose of BDO_2 would greatly improve our understanding of the contribution of specific BD metabolites in carcinogenesis.

As a result of continued methods development, globin samples are now identically processed for the detection of HBVal and THBVal up to the acetylation step for THBVal using Method II. In this way, the same sample can be split for analysis of both analytes from a single derivatization. It

is necessary to employ a GC-MS/MS instrument for the analysis of HBVal. Since the time this research was conducted, we have acquired a new Finnegan TSQ 7000 that performs GC-MS/MS and has a cooler source. The methods developed under this research project are highly suitable for measuring HBVal and THBVal in BD-exposed and unexposed individuals.

In summary, this research project has provided a new, highly sensitive method for measuring THBVal using GC and high-resolution MS. This adduct can be used as a biomarker of BD exposure in an occupational setting (and is being used in the transitional epidemiologic study of Professor Albertini being sponsored by the Health Effects Institute). The research has also pointed out that our current understanding of BD metabolism is inadequate. Past studies have focused on BDO and BDO_2 but have ignored BDO-diol. The data from this project and similar studies being conducted in our laboratory using DNA from tissues of rats and mice exposed to lower concentrations of BD strongly suggest that BDO-diol is the major electrophile produced in rats, mice, and humans. Using literature values for BD metabolites and the DNA adduct data, we were able to calculate that ~95% of the THB-guanine adducts arose from BDO-diol (Koc et al. 1999). This is also likely to be the case for THBVal adducts. Accurate assessments of cancer risk in humans from exposure to BD cannot be made until a better understanding of the metabolism of BD is gained in laboratory animals and humans. Molecular dosimetry studies on DNA and hemoglobin adducts of BD will provide critical data for developing this improved understanding.

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PUBLICATIONS RESULTING FROM THIS RESEARCH

Hayes RB, Zhang L, Yin S, Swenberg JA, Wiencke J, Bechtold WE, Yao M, Rothman N, Haas R, O'Neill PO, Wiemels J, Dosemeci M, Li G, Smith M. 1999. Genotoxic markers among butadiene-polymer workers in China. Carcinogenesis 21:55–62.

ABBREVIATIONS AND OTHER TERMS

amu	atomic mass unit
ANOVA	analysis of variance
BD-diol	1,2-dihydroxy-3-butene
BD	1,3-butadiene
BDO	1,2-epoxy-3-butene
BDO_2	1,2,3,4-diepoxybutane
BDO-diol	1,2-dihydroxy-3,4-epoxybutane
CYP2E1	cytochrome P450 2E1
DDW	distilled deionized water
DMF	dimethyl formamide
ECNCI	electron-capture negative chemical ionization
GC-MS	gas chromatography–mass spectrometry
GST	glutathione *S*–transferase
HBVal	*N*-(2-hydroxy-3-butenyl)valine
HEVal	*N*-(2-hydroxyethyl)valine
HPLC	high-pressure liquid chromatography
$KHCO_3$	potassium bicarbonate
LC-ESI-MS	liquid chromatography–negative ion electrospray–mass spectrometry
M	mass [in figures only]
MI	1,2-dihydroxy-4-(*N*-acetylcysteinyl)butane
MS/MS	tandem mass spectrometry
m/z	mass-to-charge ratio
Na_2CO_3	sodium carbonate
NaOH	sodium hydroxide
PFPITC	pentafluorophenylisothiocyanate
PFPTH	pentafluorothiohydantoin
r^2	regression coefficient
RBCs	red blood cells
SIM	selected ion monitoring
THBVal	*N*-(2,3,4-trihydroxybutyl)valine